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Huperzine A, but not tacrine, stimulates S100B secretion in astrocyte cultures

Paula Lunardi [a,b], Patrícia Nardin [b], Maria Cristina Guerra [b], Renata Abib [b], Marina Concli Leite [b], Carlos-Alberto Gonçalves [a,*]

[a] Programa de Pós-Graduação em Neurociências, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Ramiro Barcelos, 2600-Anexo, 90035-003 Porto Alegre, Brazil
[b] Programa de Pós-Graduação em Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Ramiro Barcelos, 2600-Anexo, 90035-003 Porto Alegre, Brazil

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ABSTRACT

Aims: The loss of cholinergic function in the central nervous system contributes significantly to the cognitive decline associated with advanced age and dementias. Huperzine A (HupA) is a selective inhibitor of acetylcholinesterase (AChE) and has been shown to significantly reduce cognitive impairment in animal models of dementia. Based on the importance of astrocytes in physiological and pathological brain activities, we investigated the effect of HupA and tacrine on S100B secretion in primary astrocyte cultures. S100B is an astrocyte-derived protein that has been proposed to be a marker of brain injury.
Main methods: Primary astrocyte cultures were exposed to HupA, tacrine, cholinergic agonists, and S100B secretion was measured by enzyme-linked immunosorbent assay (ELISA) at 1 and 24 h.
Key findings: HupA, but not tacrine, at 100 μM significantly increased S100B secretion in astrocyte cultures. Nicotine (at 100 and 1000 μM) was able to stimulate S100B secretion in astrocyte cultures.
Significance: Our data reinforce the idea that AChE inhibitors, particularly HupA, do not act exclusively on the acetylcholine balance. This effect of HupA could contribute to improve the cognitive deficit observed in patients, which are attributed to cholinergic dysfunction. In addition, for the first time, to our knowledge, these data indicate that S100B secretion can be modulated by nicotinic receptors, in addition to glutamate, dopamine and serotonin receptors.

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Introduction

Huperzine A (HupA), a Lycopodium alkaloid isolated from the Chinese herb **Huperzia serrata**, (Ma et al., 2007; Wang et al., 1986) is a selective inhibitor of acetylcholinesterase (AChE) and has been shown to significantly reduce cognitive impairment in animal models of dementia and in Alzheimer's disease (AD) patients in China (Wang et al., 2006, 2009). Compared with other AChE inhibitors, e.g. tacrine, HupA demonstrates a better passage through the blood–brain barrier, higher oral bioavailability, longer duration of AChE inhibition, fewer peripheral side effects, and is devoid of dose-limiting hepatotoxicity (Bai et al., 2000; Liang and Tang, 2004; Tang, 1996; Tang et al., 1994; Wang and Tang, 1998a; Xiong and Tang, 1995).

AD exhibits several neurochemical alterations including β-peptide amyloidosis, inflammation and oxidative stress, leading to neuronal population destruction, especially of cholinergic neurons (Davies and Maloney, 1976; Geula et al., 2008; Mufson et al., 2002, 2008; Verdier et al., 2004). This loss of cholinergic function in the central nervous system contributes significantly to the cognitive decline associated with advanced age and AD (Bartus, 2000; Bartus et al., 1982; Schliebs and Arendt, 2011; Terry and Buccafusco, 2003) and has justified the use of AChE inhibitors as a strategy for patients with AD (Birks, 2006; Giacobini, 2000; Nordberg and Svensson, 1998; Talesa, 2001; Whitehouse, 1993). Moreover, many other neuroprotective effects of these compounds, in addition to AChE inhibition, have been described (Nordberg, 2006; Racchi et al., 2004; Takatori, 2006). Some studies have found that HupA displays neuroprotective properties with multi-target effects (Zhang and Tang, 2006; Zhang et al., 2008a, 2008b). HupA has improved chronic inflammation and cognitive decline in diverse animal models of dementia (Wang et al., 2001, 2010; Wang and Tang, 1998b; Zhang et al., 2004; Zhou et al., 2001).

S100B, a calcium-binding protein, produced and secreted by astrocytes in the central nervous system, plays a regulatory role in the cytoskeleton and cell cycle (Donato et al., 2009). Extracellular S100B works as a neurotrophic cytokine and has been proposed as a marker for brain injury, including that seen in AD (Goncalves et al., 2008). In fact, it has been reported that this protein could affect neuronal β-amyloid protein synthesis (Li et al., 1998). On the other hand, we have observed decreased levels of this protein in the cerebrospinal fluid in rat models of dementia, including chronic cerebral hypoperfusion (Vicente et al., 2009), intracerebroventricular administration of streptozotocin (Rodrigues et al., 2009) and okadaic acid (Costa et al., 2012).

* Corresponding author at: Departamento Bioquímica, ICBS, UFRGS, Ramiro Barcelos, 2600-Anexo, 90035-003 Porto Alegre, Brazil. Fax: +55 51 3308 5535.
E-mail address: casg@ufrgs.br (C.-A. Gonçalves).

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S100B secretion appears to be modulated by antipsychotic (Nardin et al., 2011; Steiner et al., 2009) and anti-depressive drugs (Manev and Manev, 2002; Tramontina et al., 2008), commonly used for the symptomatic treatment of dementia. However, the effect of AChE inhibitors and cholinergic agonists on S100B secretion has not been investigated. Considering the importance of astrocytes in physiological and pathological brain activities (Halassa et al., 2009; Halassa and Haydon, 2010), we decided to investigate the effect of HupA and tacrine on S100B secretion astrocyte cultures using HupA at concentrations currently described in the literature (from 0.1 to 100 μM), as well as cholinergic agonists. Our results suggest, for the first time to our knowledge, a cholinergic modulation of S100B secretion.

Material and methods

Animals

Twenty newborn Wistar rats (1 or 2-days old) (used for astrocyte cultures) and twelve male 30-day old rats (used for hippocampal slice preparations) were obtained from our breeding colony (at the Department of Biochemistry, Universidade Federal do Rio Grande do Sul, Brazil). Rats were maintained under controlled light and environmental conditions (12 h light/12 h dark cycle at a constant temperature of 22 ± 1 °C) with free access to food and water. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23), revised 1996, and following the regulations of the local animal house authorities (Approval 20375).

Drugs and reagents

(−)Huperzine A, tacrine, acetylthiocholine, nicotine, mecamylamine, carbachol, scopolamine, poly-L-lysine, monoclonal anti-S100B antibody (SHB1), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), **o**-phenylenediamine (OPD) and methylthiazolyldiphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, USA). Fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM) and other materials for cell culture were purchased from Gibco (Carlsbad, USA). Polyclonal anti-S100B and anti-rabbit linked peroxidase were purchased from DAKO (São Paulo, Brazil) and GE (Little Chalfont, United Kingdom), respectively.

Astrocyte cultures

Primary astrocyte cultures from Wistar rats were prepared as previously described (Gottfried et al., 1999). Briefly, cerebral cortices of newborn Wistar rats were removed and mechanically dissociated in Ca^{2+}- and Mg^{2+}-free balanced salt solution, pH 7.4, containing (in mM): 137 NaCl; 5.36 KCl; 0.27 Na_2HPO_4; 1.1 KH_2PO_4 and 6.1 glucose. The cortices were cleaned of meninges and mechanically dissociated by sequential passages through a Pasteur pipette. After centrifugation at 1400 RPM for 5 min, the pellet was resuspended in DMEM (pH 7.6) supplemented with 8.39 mM HEPES, 23.8 mM $NaHCO_3$, 0.1% amphotericin, 0.032% gentamicin and 10% fetal calf serum. The cells were plated at a density of 1.5×10^5 cells/cm^2 in 24 well plates pre-coated with poly-L-lysine. Cultures were maintained in DMEM containing 10% FCS in 5% CO2/95% air at 37 °C, allowed to grow to confluence, and used in vitro at 15 days. Immunocytochemistry for glial fibrillary acidic protein (GFAP), a specific protein for astrocytes, indicated a cell purity of higher than 95% (data not shown). Treatments consisted of HupA (0.1, 1, 10 and 100 μM), tacrine (10 and 100 μM), acetylthiocholine (10 and 100 μM), nicotine, mecamylamine, carbachol and scopolamine, all these last four drugs were used at 1–1000 μM, for 1 and 24 h at 37 °C. Drug concentrations were chosen based on pilot experiments. All experiments were performed in triplicate. Afterwards, the collected culture medium and the cell lysates were stored at −20 °C until used in assays for S100B.

Hippocampal slices

Hippocampal slices were prepared as previously described (Nardin et al., 2009). Thirty-day old Wistar rats were killed by decapitation and the brains were removed and placed in cold saline medium with the following composition (in mM): 120 NaCl; 2 KCl; 1 $CaCl_2$; 1 $MgSO_4$; 25 HEPES; 1 KH_2PO_4, and 10 glucose, adjusted to pH 7.4 and previously aerated with O_2. The hippocampi were dissected and transverse slices of 0.3 mm were obtained using a McIlwain Tissue Chopper. Slices were then transferred immediately to 24-well culture plates, each well containing 0.3 ml of physiological medium and only one slice. The medium was changed every 15 min for 2 h at room temperature. After this stabilization period, the drugs were added and the slices incubated for 1 h at 30 °C. Treatments were the same as those used in astrocyte cultures.

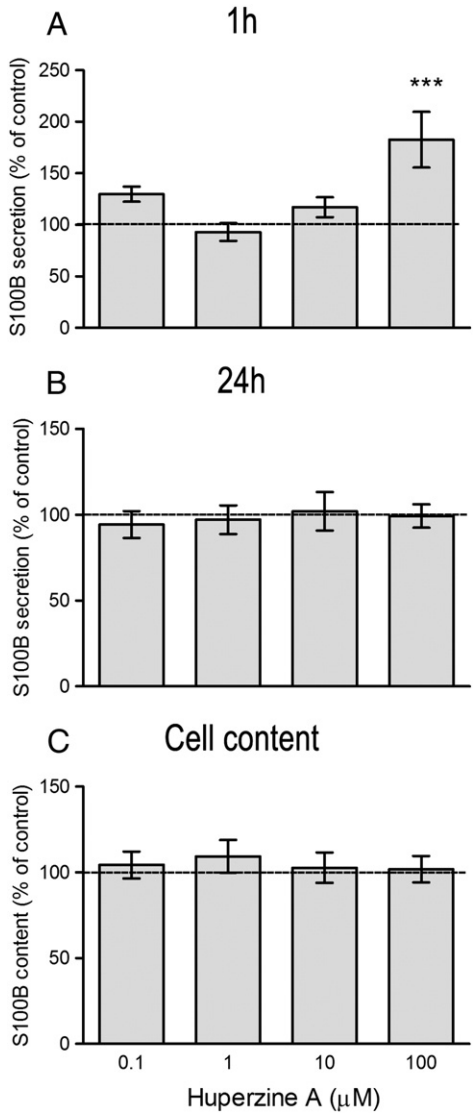

Fig. 1. *Effect of Huperzine A (HupA) on S100B secretion and content*. Astrocyte cultures were incubated with HupA (0.1, 1, 10 and 100 μM) and the S100B levels in the medium were measured for 1 h (A) and 24 h (B), as well as the S100B content (C) by ELISA. Each value is the mean (± standard error) of five independent experiments performed in triplicate, expressed as a percentage of the control (indicated by dashed line). ***$p<0.001$ compared to control (one-way ANOVA followed by Dunnet's post-hoc assay).

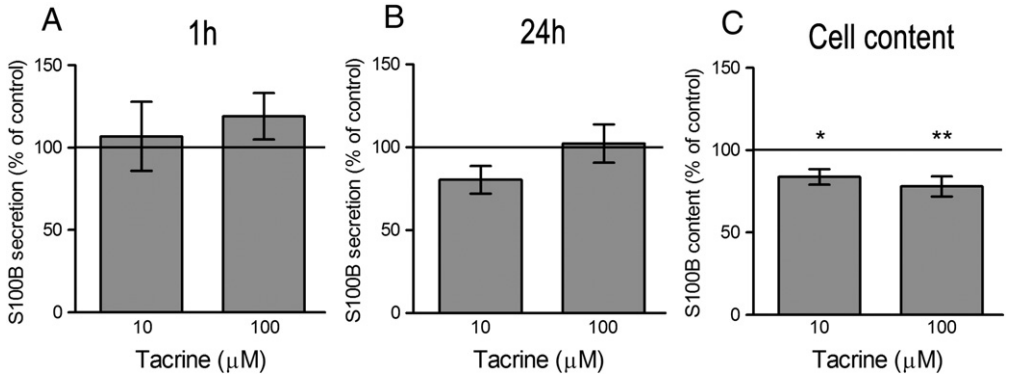

Fig. 2. *Effect of tacrine on S100B secretion and content.* Astrocyte cultures were incubated with tacrine (10 and 100 μM). S100B levels in the medium were measured for 1 h (A) and 24 h (B), as well as the S100B content (C) by ELISA. Each value is the mean (± standard error) of five independent experiments performed in triplicate, expressed as a percentage of the control (indicated by line). *$p<0.05$ and **$p<0.01$ compared to control (one-way ANOVA followed by Dunnet's post-hoc assay).

S100B measurement

S100B levels were measured by enzyme-linked immunosorbent assay (ELISA), as previously described (Leite et al., 2008). Briefly, 50 μl of sample plus 50 μl of Tris buffer were incubated for 2 h in a microtiter plate previously coated with monoclonal anti-S100B. Polyclonal anti-S100 was incubated for 30 min and then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min. The color reaction with **o**-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.002 to 1 ng/ml.

Cell viability and integrity

Cell viability was assayed by the colorimetric MTT reduction method (Hansen et al., 1989) and by neutral red incorporation (Leite et al., 2009). For the MTT reduction, cells were incubated with 0.5 mg/ml of MTT at 37 °C for 30 min. The formazan product generated during the incubation was solubilized in dimethyl sulfoxide (DMSO). Absorbance values were measured at 560 and 630 nm. The neutral red assay was carried out by treating cells with 50 μg/ml neutral red (NR) at 37 °C for 30 min. Afterwards, the cells were rinsed twice with PBS for 5 min each and NR dye taken up by viable cells was extracted with acetic acid/ethanol/water (1/50/49). Absorbance values were measured at 560 nm. Cell integrity was indicated by lactate dehydrogenase (LDH) activity in the incubation medium. Determination was carried out by a colorimetric commercial kit (Doles Reagentes e Equipamentos para Laboratórios Ltda., Brazil), according to the manufacturer's instructions. Results were expressed as percentages of the control.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Dunnet's post hoc test. Results represent mean ± S.E.M. GraphPad Prism 5 for Windows was used for statistical analysis; $p<0.05$ was set as statistically significant, and values of significance were indicated by different symbols: one asterisk (*) for $p<0.05$; two asterisks (**) for $p<0.01$; and three asterisks (***) for $p<0.001$.

Table 1
Viability and integrity of astrocyte cultures and hippocampal slices treated with HupA, at 10 or 100 μM, for 24 h and 1 h, respectively.

	Astrocyte cultures		Hippocampal slices	
	NR	LDH	NR	LDH
HupA (10 μM)	100.0 ± 7.7	114.2 ± 9.3	119.2 ± 1.3	99.0 ± 7.3
HupA (100 μM)	110.3 ± 9.5	105.8 ± 7.0	123.1 ± 4.8	94.1 ± 1.4

NR, neutral red uptake; LDH, lactate dehydrogenase activity. Data are expressed as percentage of control and represent mean ± S.E.M.

Results

Huperzine A, but not tacrine, stimulates S100B secretion in astrocyte cultures

We investigated S100B secretion in astrocyte cultures by adding concentrations of HupA from 0.1 to 100 μM (Fig. 1A). HupA at 100 μM induced an increase in S100B secretion at 1 h ($F_{4,\ 25} = 6.608$, $p = 0.0009$). However, no changes were observed in secretion (Fig. 1B) or in the intracellular S100B content (Fig. 1C) at 24 h afterwards. In contrast, tacrine at 10 or 100 μM was not able to alter S100B secretion at 1 h (Fig. 2A) and at 24 h (Fig. 2B). Nevertheless, tacrine at both concentrations was able to reduce the intracellular content of S100B at 24 h (Fig. 2C) ($F_{2,\ 14} = 7.058$, $p = 0.0076$).

Huperzine A exposure did not affect cell integrity or viability

In order to evaluate cell integrity and viability of astrocytes exposed to HupA for 24 h, at two higher concentrations (10 and 100 μM), we carried out 3 assays: LDH activity, neutral red assay and MTT reduction assay. No significant differences were observed in extracellular LDH activity, suggesting that cell integrity was preserved during HupA exposure (Table 1). Two other assays for cell viability, neutral red assay (Table 1) and MTT reduction assay (data not shown), were not modified by HupA.

Acetylthiocholine, like Huperzine A, stimulated S100B secretion in astrocyte cultures

Acetylthiocholine is the substrate used for measurement of AChE activity and also works as a cholinergic agonist. This compound, at 100 μM, increased S100B secretion in astrocyte cultures at 1 h (Fig. 3A) ($F_{2,\ 13} = 21.04$, $p = 0.0001$). No changes in S100B secretion (Fig. 3B) or S100B content (Fig. 3C) were observed at 24 h.

Cholinergic modulation of S100B secretion in astrocyte cultures

Results for tacrine and acetylthiocholine suggest that the effect of HupA on S100B secretion was independent of AChE inhibition and possibly involves cholinergic regulation. Therefore, we investigated the involvement of cholinergic modulation in basal S100B secretion in astrocyte cultures. Four compounds were used: nicotine, mecamylamine (a nicotinic antagonist), carbachol (a muscarinic agonist) and scopolamine (a muscarinic antagonist), at concentrations from 1 to 1000 μM.

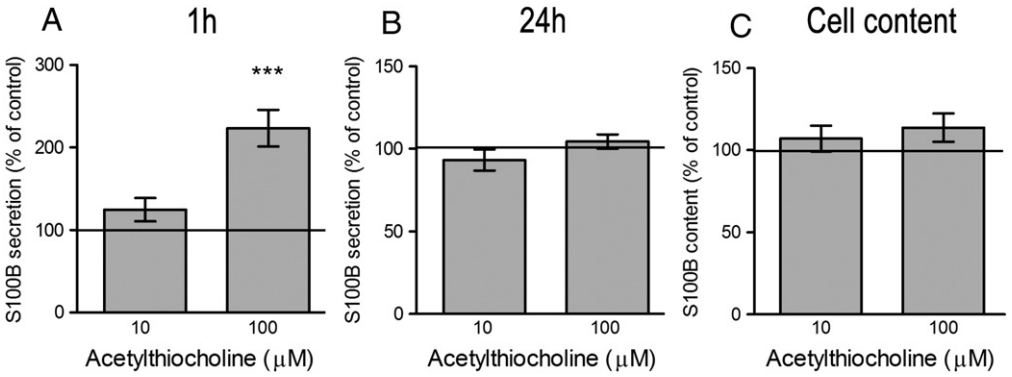

Fig. 3. *Effect of acetylthiocholine on S100B secretion and content.* Astrocyte cultures were incubated with acetylthiocholine (10 and 100 μM). S100B levels in the medium were measured for 1 h (A) and 24 h (B), as well as the S100B content (C) by ELISA. Each value is the mean (± standard error) of five independent experiments performed in triplicate, expressed as a percentage of the control (indicated by line). ***p<0.001 compared to control (one-way ANOVA followed by Dunnet's post-hoc assay).

When nicotine at 100 or 1000 μM was added to astrocyte cultures (Fig. 4A), we observed an increase in S100B secretion at 1 h ($F_{4, 46}$ = 5.134, p = 0.0017). No changes were observed in S100B secretion (Fig. 4B) or S100B intracellular content (Fig. 4C) at 24 h. Expectedly, mecamylamine was not able to change S100B secretion at 1 h (Fig. 5A), but increased S100B secretion at 24 h (Fig. 5B) ($F_{4, 22}$ = 12.30, p = 0.0001). No changes, induced by mecamylamine, were observed in the S100B intracellular content (data not shown). Mecamylamine (at 1 mM) blocked the increase in S100B secretion induced by HupA, and also blocked the effect of nicotine (Fig. 5C) ($F_{3, 33}$ = 9.519, p = 0.0001). No changes in S100B secretion or content were observed when carbachol or scopolamine was added to astrocyte cultures (data not shown).

S100B secretion was reduced by Huperzine A and acetylthiocholine in fresh hippocampal slices

Finally we evaluated the effect of HupA on basal S100B secretion in acute hippocampal slices. Surprisingly, HupA at 100 μM decreased S100B secretion in this preparation (Fig. 6A) ($F_{4, 53}$ = 5.165, p = 0.0014). This effect also occurred with acetylthiocholine at 100 μM (Fig. 6B, $F_{2, 22}$ = 12.51, p = 0.0002), but not with tacrine at 100 μM (data not shown). Nicotine at 100 and 1000 μM (Fig. 6C) did not significantly change S100B secretion, but at lower concentrations (1 and 10 μM) we observed an increase in S100B secretion ($F_{4, 37}$ = 3.628, p = 0.0136).

Discussion

HupA has been used in schizophrenia and AD in China and has been proposed as a selective, reversible, and well-tolerated inhibitor of AChE, and an even more potent inhibitor of AChE than tacrine, donepezil, rivastigmine or galanthamine in vivo (Ma et al., 2007; Wang et al., 2009). However, most of the clinical trials that have employed this compound have been performed in China and this substance has not been approved by the Food and Drug Administration (FDA) in the USA or by the National Agency of Sanitary Vigilance (ANVISA) in Brazil yet. As such, the characterization of some putative effects of this compound on some neurochemical targets is necessary.

We herein analyzed the effect of HupA on S100B secretion, assumed to be a marker of activation of astrocytes (Donato et al., 2009; Goncalves et al., 2008), which play an important role in both physiological and pathological brain processes (Halassa and Haydon, 2010; Rodriguez et al., 2009; Verkhratsky, 2006). Recent studies have emphasized the role of astrocytes (via nicotinic and muscarinic receptors) in cholinergic neurotransmission and long-term potentiation (LTP) (Navarrete et al., 2012; Shen and Yakel, 2012). Moreover, a role of extracellular S100B in LTP has been previously reported (Nishiyama et al., 2002).

Our results indicate that HupA is able to induce an increase in S100B secretion in cortical astrocytes in culture, but a decrease in S100B secretion in acute hippocampal slices. These opposing effects

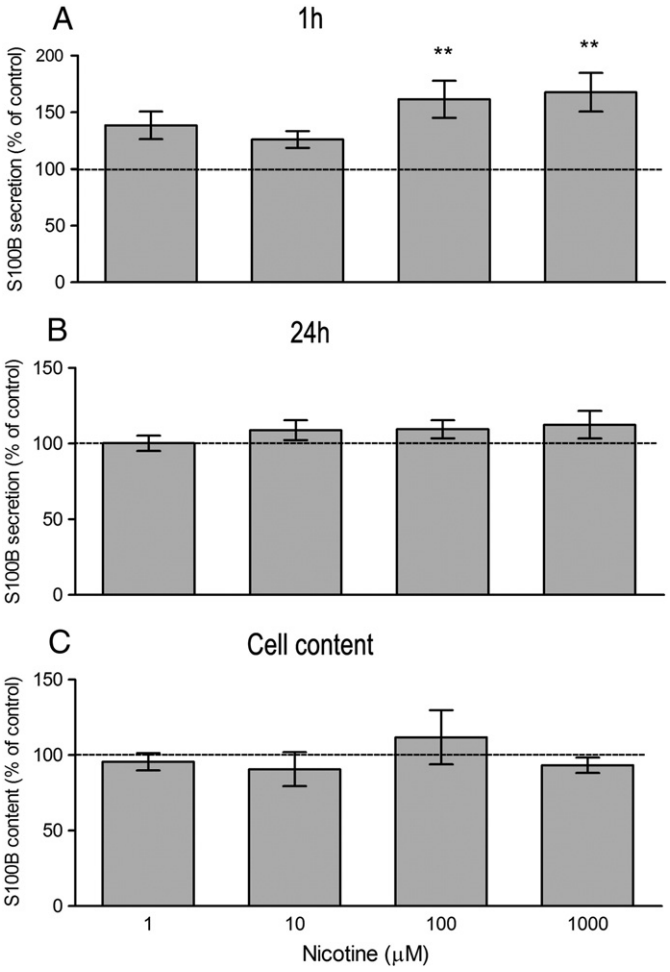

Fig. 4. *Effect of nicotine-induced S100B secretion and content.* Astrocyte cultures were incubated with nicotine (1, 10, 100 and 1000 μM) and the S100B levels in the medium were measured for 1 h (A) and 24 h (B), as well as the S100B content (C) by ELISA. Each value is the mean (± standard error) of five independent experiments performed in triplicate and expressed as a percentage of the control (indicated by dashed line). **p<0.01 compared to control (one-way ANOVA followed by Dunnet's post-hoc assay).

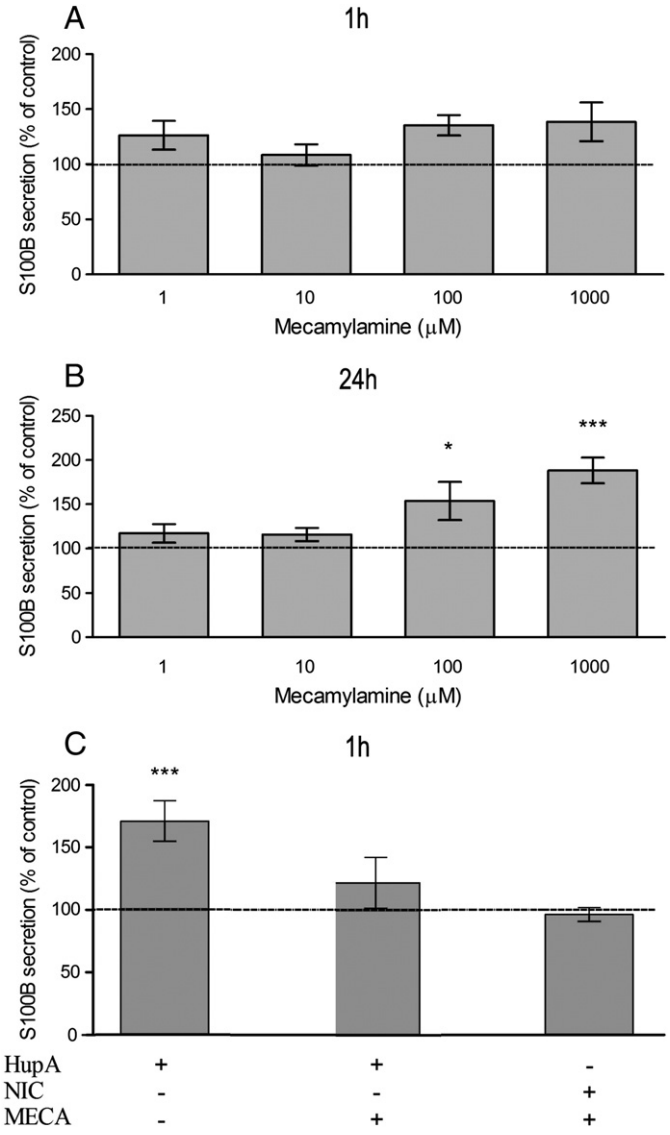

Fig. 5. *Effect of mecamylamine-induced S100B secretion and content*. Astrocyte cultures were incubated with mecamylamine (1, 10, 100 and 1000 μM) and the S100B levels in the medium were measured for 1 h (A) and 24 h (B). In C, astrocyte cultures were incubated with mecamylamine (MECA, at 1 mM) and nicotine (NIC, at 1 mM) or Huperzine A (HupA, at 100 μM) for 1 h. S100B levels in the medium were measured by ELISA. Each value is the mean (± standard error) of five independent experiments performed in triplicate and expressed as a percentage of the control (indicated by dashed line). *$p<0.05$ and ***$p<0.001$ compared to control (one-way ANOVA followed by Dunnet's post-hoc assay).

have also been described in other studies; for example, endothelin-1 induces S100B secretion in astrocyte cultures, but a decrease in hippocampal slices (Leite et al., 2009). Such discrepancies can be attributed to differences in cell signaling between isolated cells and the complex cell environment of slice preparation and/or the heterogeneity of astrocyte populations from different brain regions. Moreover, under our conditions, no differences were found in relation to cell integrity or viability in either preparation.

Our results regarding the increase in S100B secretion in cortical astrocytes suggest a trophic role of HupA, based on the in vitro neurotrophic activity of S100B (Donato et al., 2009; Serbinek et al., 2010) and based on the acute administration of this protein in vivo after brain injury (Kleindienst and Ross Bullock, 2006). In contrast, tacrine was not able to induce S100B secretion in astrocyte cultures (or a decrease in hippocampal slices). This result suggests that the modulation of S100B secretion by HupA is independent of AChE inhibition, but does not rule out a possible effect of HupA on cholinergic neurotransmission.

Confirming this possibility, a similar effect to that of HupA on S100B secretion was also obtained with acetylthiocholine, a cholinergic agonist, in both cortical astrocytes and hippocampal slices. Nicotine was also able to induce S100B secretion in astrocyte cultures (at concentrations of 100 and 1000 μM) and in hippocampal slices (at concentrations of 1 and 10 μM). The Huperzine A or nicotine-induced S100B secretion in astrocyte cultures was blocked by mecamylamine, a non-selective nicotinic antagonist. Moreover, in astrocyte cultures mecamylamine did not affect basal S100B secretion at 1 h, but was able to increase S100B secretion 24 h later. No effect on acute basal S100B secretion was observed with the addition of carbachol, a muscarinic agonist, or scopolamine, a muscarinic antagonist. Therefore, these data suggest a specific involvement of nicotinic receptors in the modulation of S100B secretion in astrocyte cultures.

The mechanism by which HupA affects S100B secretion remains unclear, but we may make some speculations regarding the involvement of cholinergic and/or NMDA receptors. Studies on the displacement of $[H^3]$QNB- and $[H^3](-)$nicotine-specific binding showed that HupA has a direct effect on cholinergic receptors (Tang et al., 1989). However, there are no data about binding or the direct action of HupA on cholinergic presynaptic receptors and the control of acetylcholine release (Wang et al., 2006). On the other hand, HupA was able to block the release of TNF-alpha, an inflammatory cytokine, in cultures of microglial cells submitted to hypoxia, and this effect was antagonized by mecamylamine (Wang et al., 2012). In agreement with this, our data reinforce the involvement of nicotinic receptors in HupA activity. Moreover, it has been proposed that HupA acts as an NMDA antagonist (Gordon et al., 2001). This possibly should be investigated, however NMDA was unable to induce S100B secretion in astrocyte cultures (Goncalves et al., 2002).

As mentioned before HupA has also been used in the treatment of schizophrenia in China (Ma et al., 2007). Antipsychotic drugs (putatively acting on dopamine receptors) are reported to modulate S100B secretion (Nardin et al., 2011; Steiner et al., 2009). Therefore, it would be interesting to evaluate whether the modulation of S100B secretion by HupA also involves dopamine receptors.

Conclusions

Our data reinforce the idea that AChE inhibitors, particularly HupA, do not act exclusively on the acetylcholine balance. HupA, per se, could be acting via nicotinic receptors, at least with regards to astroglial S100B secretion. This action could contribute to improve the cognitive deficit observed in AD patients, which is attributed to cholinergic dysfunction. This reinforces the importance of astrocytes as targets of neuroactive compounds in brain diseases. In addition, for the first time, to our knowledge, these data indicate that S100B secretion can be modulated by nicotinic receptors, in addition to glutamate, dopamine and serotonin receptors.

Conflict of interest statement

There is no conflict of interest.

Acknowledgments

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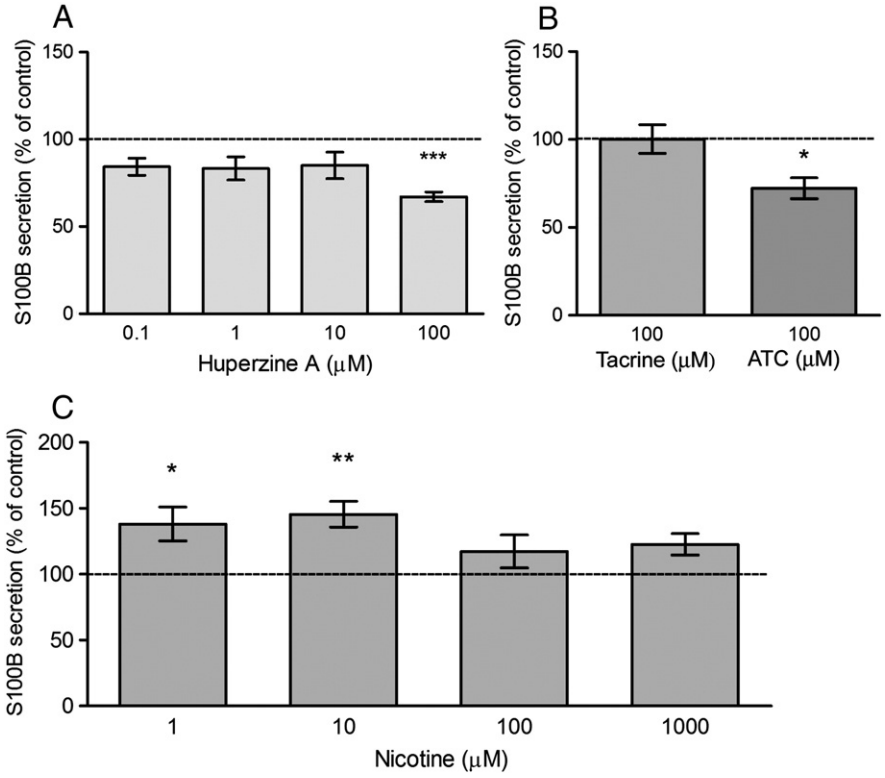

Fig. 6. *Effect of Huperzine A, tacrine, acetylthiocholine and nicotine on S100B secretion in hippocampal slices.* Hippocampal slices were incubated with HupA (0.1, 1, 10 and 100 μM) (A), tacrine and acetylthiocholine (ATC) at 100 μM (B) and nicotine (1, 10, 100 and 1000 μM) (C). S100B levels in the medium were measured for 1 h by ELISA. Each value is the mean (± standard error) of six independent experiments performed in triplicate, expressed as a percentage of the control (indicated by dashed line). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to control (one-way ANOVA followed by Dunnet's post-hoc assay).

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